

Basic Study

S-adenosyl-L-methionine modifies antioxidant-enzymes, glutathione-biosynthesis and methionine adenosyltransferases-1/2 in hepatitis C virus-expressing cells

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Abstract

AIM: To elucidate the mechanism(s) by which S-adenosyl-L-methionine (SAM) decreases hepatitis C virus (HCV) expression.

METHODS: We examined the effects of SAM on viral expression using an HCV subgenomic replicon cell culture system. Huh7 HCV-replicon cells were treated with 1 mmol/L SAM for different times (24-72 h), then total RNA and proteins were isolated. cDNA was synthesized and real time-PCR was achieved to quantify HCV-RNA, superoxide dismutase 1 and 2

(SOD-1, SOD-2) catalase, thioredoxin 1, methionine adenosyltransferase 1A and 2A (MAT1A, MAT2A) expression, and GAPDH and RPS18 as endogenous genes. Expression of cellular and viral protein was evaluated by western-blot analysis using antibodies vs HCV-NS5A, SOD-1, SOD-2, catalase, thioredoxin-1, MAT1A, MAT2A, GAPDH and actin. Total glutathione levels were measured at different times by Ellman's recycling method (0-24 h). Reactive oxidative species (ROS) levels were quantified by the dichlorofluorescein assay (0-48 h); Pyrrolidin dithiocarbamate (PDTC) was tested as an antioxidant control and H₂O₂ as a positive oxidant agent.

RESULTS: SAM exposition decreased HCV-RNA levels 50%-70% compared to non-treated controls (24-72 h). SAM induced a synergic antiviral effect with standard IFN treatment but it was independent of IFN signaling. In addition, 1 mmol/L SAM exposition did not modify viral RNA stability, but it needs cellular translation machinery in order to decrease HCV expression. Total glutathione levels increased upon SAM treatment in HCV-replicon cells. Transcriptional antioxidant enzyme expression (SOD-1, SOD-2 and thioredoxin-1) was increased at different times but interestingly, there was no significant change in ROS levels upon SAM treatment, contrary to what was detected with PDTC treatment, where an average 40% reduction was observed in exposed cells. There was a turnover from MAT1A/MAT2A, since MAT1A expression was increased (2.5 fold-times at 48 h) and MAT2A was diminished (from 24 h) upon SAM treatment at both the transcriptional and translational level.

CONCLUSION: A likely mechanism(s) by which SAM diminish HCV expression could involve modulating antioxidant enzymes, restoring biosynthesis of glutathione and switching MAT1/MAT2 turnover in HCV expressing cells.

Key words: Hepatitis C virus; S-adenosyl-L-methionine; Superoxide dismutase 1; Superoxide dismutase 2; Replication; Hepatitis C virus-RNA; NS5A; Oxidative stress; Antioxidants; Viral proteins; Reactive oxygen species; Pyrrolidine dithiocarbamate

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Core tip: S-adenosyl-L-methionine (SAM) downregulates hepatitis C virus (HCV) expression by unknown mechanisms. We evaluated the effects of SAM on viral expression using an HCV subgenomic replicon cell culture system. We observed that SAM induces a synergic antiviral effect with standard interferon (IFN) treatment independently of IFN signaling pathways; it does not modify viral RNA stability, but it needs cellular translation machinery in order to decrease HCV expression. In addition, results demonstrated that a likely mechanism(s) by which SAM decreases HCV expression could involve modulating antioxidant

enzyme systems, restoring biosynthesis of glutathione and switching methionine adenosyltransferase 1 (MAT1)/MAT2 turnover in HCV expressing cells.

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INTRODUCTION

Chronic hepatitis C (CHC) is a global burden^[1]; around 3% (170-200 million people) of the total population is infected with hepatitis C virus (HCV)^[2] and CHC eventually may lead to cirrhosis and hepatocellular carcinoma (HCC)^[3]. In over 70% of cases, the immune response is unable to clear the virus, resulting in viral persistence. Current standard of care is based on the mixed scheme of pegylated interferon 2 α (PEG-IFN), ribavirin (RBV)^[4] and a protease or a polymerase inhibitor^[5].

The mechanisms by which HCV causes cell damage are poorly elucidated and different metabolic pathways have been proposed in its pathogenesis, including the active participation of oxidative stress in chronic HCV infection. A major increase in several markers of oxidative stress are reported in HCV infection, such as inflammation, iron overload and mitochondrial injury, induced by viral proteins^[6]. Oxidative stress has been described to be present in CHC patients. To date, reactive oxygen species (ROS) induction has been a subject of study for understanding HCV pathogenesis^[7]. Direct measures in liver tissue show that patients with CHC have an increase in ROS^[8]. Cells get protection against oxidative damage by natural antioxidant molecules, notably glutathione, and several antioxidant enzymes, such as superoxide dismutases (SOD1 and SOD2), catalase (CAT), glutathione peroxidase (GPx) and thioredoxin 1 (TRX1)^[9]. Studies in subjects with chronic HCV infection have found decreased levels of glutathione^[10,11] and other antioxidants and enzymatic activity^[12-14]. Levent *et al*^[15], published a clinical study in which different oxidative stress markers were measured in CHC patients, showing that plasmatic SOD1 levels had a significant reduction compared with healthy subjects. A reduction in SOD1 and GPx reflects a decrease in liver synthesis and antioxidant power in patients with HCV, and this might be considered an early marker of oxidative stress. At the same time, when markers of oxidative stress were compared in patients infected with HCV, before and after treatment, the levels showed a recovery^[15]. However, more data

are needed in order to understand the role of HCV expression in the disturbance of oxidative stress levels in infected cells.

Recently, it has been reported that S-adenosyl-L-methionine (SAM), decreases HCV RNA levels; however, the mechanism(s) involved are unknown. SAM is the main precursor of glutathione synthesis. Methionine adenosyltransferase 1A (MAT1A) enzyme is responsible of its biosynthesis in normal liver, but it is replaced by MAT2A in liver regeneration and hepatic carcinoma^[16-18]. Based on this, we evaluated the effects of SAM exposition on HCV viral replication and protein expression and analyzed possible mechanisms involved using a hepatoma cell line expressing HCV proteins. Furthermore, the regulation of MAT1A and MAT2A enzymes involved in endogenous SAM synthesis was analyzed. Our findings suggest that SAM is able to diminish HCV expression at least in part through modulation of antioxidant enzymes, biosynthesis of glutathione and switching MAT1A/MAT2A turnover in HCV expressing cells. These findings suggest the possibility that SAM modulation could help to counteract HCV-induced damage.

MATERIALS AND METHODS

Cell culture and treatments

We used a genotype 1b HCV subgenomic Huh7-replicon cell culture system (harboring the subgenomic HCV replicon I389/NS3-3), described previously^[19,20]. Huh7-replicon cells were cultured in advanced Dulbecco's modified Eagle's medium (GIBCO-BRL; Grand Island, NY, United States) supplemented with 2% fetal bovine serum, 1% nonessential amino acids, 200 IU of penicillin G and 200 mg of streptomycin/mL at 37 °C. For each experimental condition, cells were plated and 24 h later, were treated either with SAM (1-5 mmol/L), a combined treatment with PEG-IFN α + RBV (double therapy), or 5 μ mol/L PDTC (time zero) and then incubated until 72 h. Cell viability was calculated by comparing the different conditions with untreated cells (100% viability). Upon each experimental condition, total RNA and proteins were extracted from the cell cultures and subjected to real-time reverse transcriptase polymerase chain reaction (RT-qPCR) and western blot analysis, respectively. S-(5'-Adenosyl)-L-methionine *p*-toluenesulfonate (SAM) salt was purchased from Sigma (St. Louis MO, United States).

RNA extraction

Upon each experimental condition, total RNA was isolated from cells using Trizol reagent (Life Technologies, Carlsbad, CA, United States) as described by the manufacturer's specification^[21]. Isolated RNA was washed with 75% ethanol and diluted in 30 μ L of RNase-free water.

Quantitative real-time RT-qPCR for HCV-RNA and mRNA of antioxidant enzymes

Total RNA was then subjected to RT using a high-capacity complementary DNA (cDNA) archive kit (Applied Biosystems, Foster City, CA, United States) as described by the manufacturer's specifications. Two hundred ng of cDNA were used to perform real-time PCR for HCV-RNA, antioxidant genes, MAT1A, MAT2A, RPS18 and GAPDH mRNA quantification. HCV amplifications were done in triplicate with the following oligonucleotides: HCV-Forward (+75-93 nt), 5'-GCGTCTAGCCATGGCGTTA-3'; HCV-Reverse (+138-157 nt), 5'-GGTTCCGCAGACCACTATGG-3'; and the TaqMan probe (+94-110 nt), 5-FAM-CTGCACGACACTCATAC-NFQ-3'. For each PCR reaction, 10 μ L of TaqMan PCR Master Mix, 1 μ L of 20 \times Assay Mix, and 9 μ L of cDNA diluted in RNase free-water were used. For cellular genes analyzed, Sybr green chemistry was used for detection, and the following oligonucleotides were used: for SOD-1 gene forward 5'-AGGGCATCATCAATTTTCGAG-3', reverse 5'-TGCCTCTCTTCATCCTTTGG-3'; SOD-2, forward 5'-GGAACGGGGACACTTACAAA-3', reverse 5'-CACCTCGTGCGAATGATGG-3'; Thioredoxin-1 forward 5'-CTGCTTTTCAGGAAGCCTTG-3', reverse 5'-TGTTGGCATGCATTTGACTT-3'; MAT1A forward 5'-ACATCGGAGTCTGTGGGAGA-3', reverse 5'-CGGTCTTGACACTGTCTCA-3'; MAT2A forward 5'-GGTAGCCTTGGAGCAACAGT-3', reverse 5'-CTGGTCTCCAGCACCAATGT-3'; CAT forward 5'-TAAGACTGACCAGGGCATC-3', reverse 5'-CAAACCTTGGTGAGATCGAA-3'; Ribosomal-18S (RPS18) forward 5'-TGTGGTGTGAGGAAAGCAG-3', reverse 5'-AAGTGACGCAGCCCTCTATG-3'. For each PCR reaction, 10 μ L of SYBR-Green PCR Master Mix, 1 μ L of each oligonucleotide 10 μ mol/L, and 8 μ L of cDNA diluted in RNase-free water were added. Conditions for temperature cycling were: setup at 50 °C for 2 min, then 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Data were recorded and plots were generated. GAPDH and RPS18 expression was monitored and used to normalize assays. For GAPDH-RNA expression, we used a GAPDH (20 \times) assay (Applied Biosystems, Foster City, CA, United States) as described by the manufacturer's specifications.

Protein extraction and immunoblot assay

After each treatment, cells were lysed and proteins were extracted and quantified as we described before^[22]. Fifty micrograms of protein were resolved by 12% SDS-PAGE and transferred onto PVDF membranes (Amersham Biosciences, Freiburg, Germany). Membranes were incubated with one of the following antibodies: anti-NS5A antibody (Abcam, Cambridge, MA, United States), anti-MAT1, anti-MAT2, anti-PKR, anti-STAT1 (Abcam, Cambridge, MA, United States)

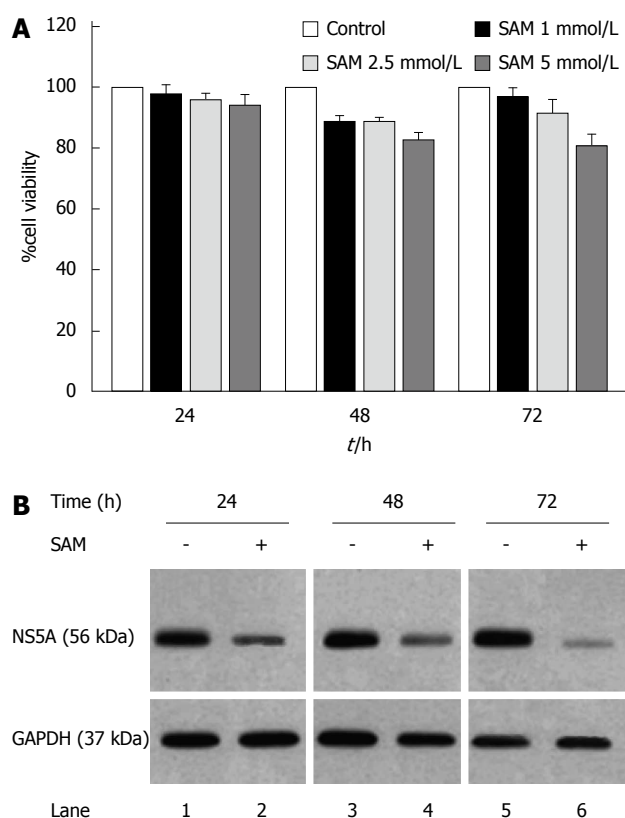


Figure 1 Effect of S-adenosyl-L-methionine in hepatitis C virus expression and cell viability. A: Cell viability in HCV-replicon cells exposed to SAM. Huh7 HCV replicon cells were treated with SAM at indicated concentrations for 24, 48 and 72 h. Cell viability was then determined by MTT assay and the % viability was calculated by comparing the different conditions with untreated cells (100% viability); B: HCV NS5A protein levels in Huh7 HCV replicon cells treated with SAM. Huh7 HCV replicon cells (2×10^5 cells) were incubated with SAM 1 mmol/L. Cell lysates were prepared, and equal amounts of protein extracts (30 μ g) were subjected to immunoblot analysis to detect NS5A. GAPDH was used as a control. SAM: S-adenosyl-L-methionine; HCV: Hepatitis C virus.

or anti-actin MAb (MP Biomedicals, Aurora, OH, United States). Upon washing with TBS/Tween, the membranes were exposed to horseradish-peroxidase conjugated anti-mouse IgG, anti-rabbit IgG or anti-goat IgG (Promega, Madison, WI, United States). Detection was made using Western-Blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotech). We performed each experiment in triplicate, and densitometric analysis was done to quantify relative expression of each protein detected by immunoblotting. The ratios of NS5A and actin were quantified with ImageJ 1.46r 2011 software. ANOVA was used to determine statistical differences. The differences were considered significant if $P < 0.05$.

Total GSH and GSSG

To determine oxidative stress levels in Huh7-replicon cells upon SAM treatment, two major indicators were evaluated at different time points and concentrations: glutathione levels and ROS production. The detection of GSH and GSSG was performed using a specific kit (GSH Assay Kit; Ann Arbor, MI, United States). Huh7

HCV-replicon and parental cells were exposed with 1 mmol/L SAM for 1, 2, 6, 12 and 24 h. Cells were disrupted with freeze and unfreeze cycles. Supernatant was collected for the analysis and stored at -80°C until the assay was done. The supernatants were low in protein (< 1 mg/mL) and were devoid of particulates so they were assayed directly without deproteinization, according to the manufacturer indications. GSSG was quantified by derivatizing GSH with 2-vinyl pyridine. The xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, United States) was used for the absorbance measure using a 415 nm filter.

ROS level quantification.

Huh7 HCV replicon cells (2×10^4 cells) were incubated with 1 mmol/L SAM at different time points (0.5, 1, 3, 12, 24 and 48 h). ROS levels were assessed by DCFH-DA assay. Fluorescence was detected at 503 nm and 530 nm, excitation and emission wavelengths respectively, by GloMax®-Multi Microplate Multimode Reader (Promega, Fitchburg, WI, United States). Hydrogen peroxide (H_2O_2 , 1 μ mol/L) was used as a positive damage control and pyrrolidine dithiocarbamate (PDTC, 5 μ mol/L) as antioxidant control.

Statistical analysis

All variables were evaluated in triplicate and experimental conditions were performed at least three times. All values were scored as means \pm SD. One-way analysis of variance was done to evaluate for differences in means and the *t*-test was performed for comparisons. In all experiments, if $P < 0.05$, the differences were considered significant.

RESULTS

SAM treatment downregulates HCV expression

First, cell viability experiments demonstrated that there were no cytotoxic effects of SAM at the concentrations of 2.5 mmol/L or less on HCV-replicon cells as demonstrated by MTT assay (Figure 1A). Also, as we previously reported, there were no cytotoxic effects of PDTC at the concentrations used. Based on this, we evaluated the effect of SAM on HCV-expression in HCV-replicon cells. We incubated cells with 1 mmol/L SAM at three different time points (24, 48 and 72 h), then cells were lysed and total proteins were extracted and subjected to western blot analysis. We observed that SAM dramatically inhibited HCV-NS5A protein levels compared with untreated cells (around 90% inhibition). In addition, this effect was time dependent because we observed a higher viral protein decrease in SAM-treated cells at 72 h post-treatment (Figure 1B). To determine if the effect of SAM on viral replication was due to the cytotoxic effect on treated cells, we evaluated cell viability and total cell count on SAM-treated cells. Figure 1A demonstrates that

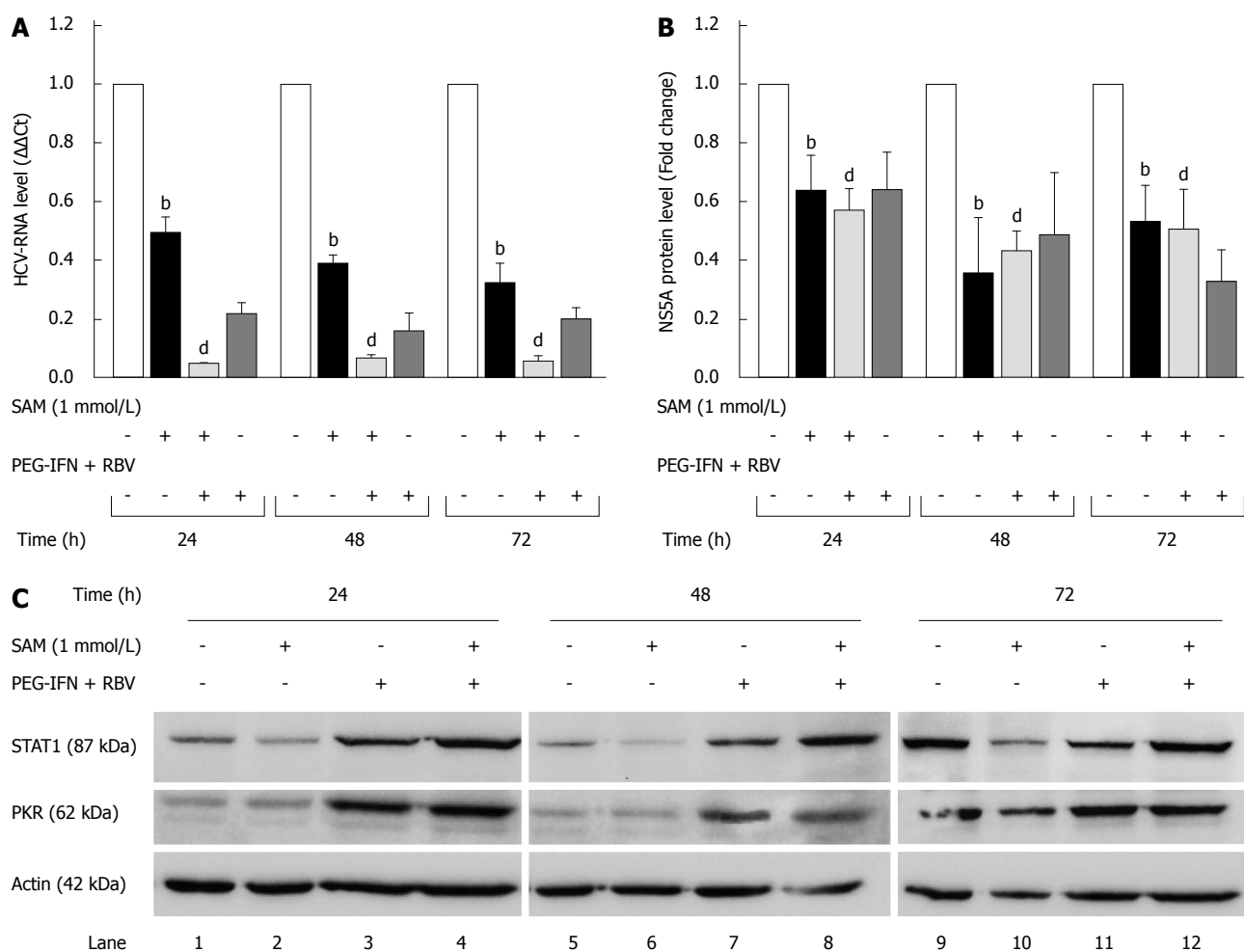


Figure 2 Synergistic antiviral effect of S-adenosyl-L-methionine plus standard interferon treatment. A: Effect of SAM and PEG-IFN + RBV on HCV-RNA levels. Huh7 HCV replicon cells (2×10^5 cells) were incubated with 1 mmol/L SAM alone or SAM plus PEG-IFN (1000 UI/mL) and RBV (50 μ mol/L). Cells were harvested at 24, 48 and 72 h and HCV-RNA levels were quantified by real-time RT-PCR and normalized with GAPDH and RPS18 using delta delta Ct method. Mean results from three independent experiments are shown. ^b $P \leq 0.01$ comparison of SAM vs untreated cells; ^a $P \leq 0.01$ comparison of SAM + PEG-IFN + RBV vs untreated cells; B: HCV NS5A protein levels in Huh7 HCV replicon cells treated with SAM PEG-IFN + RBV. Huh7 HCV replicon cells (2×10^5 cells) were incubated with 1 mmol/L SAM alone or SAM plus PEG-IFN (1000 UI/mL) and RBV (50 μ mol/L). Cell lysates were prepared, and equal amounts of protein extracts (40 μ g) were subjected to immunoblot analysis to detect NS5A and actin levels. The ratios of NS5A and actin were quantified with ImageJ 1.46r 2011 software, means of three blots are represented in the graphic. ^b $P \leq 0.01$ comparison of SAM vs untreated cells; ^a $P \leq 0.01$ comparison of SAM + PEG-IFN + RBV vs untreated cells; C: Huh7 HCV replicon cells (1.5×10^4 cells) were incubated with 1 mmol/L SAM alone, or SAM plus PEG-IFN (1000 UI/mL) and RBV (50 μ mol/L). Cells were harvested at 24, 48 and 72 h and total proteins were obtained. Cell lysates were prepared, and equal amounts of protein extracts (40 μ g) were subjected to immunoblot analysis to detect STAT1, PKR and actin levels. SAM: S-adenosyl-L-methionine; HCV: Hepatitis C virus; IFN: Interferon; RBV: Ribavirin.

no significant difference in cell number and viability was present among unexposed (100% viability) and exposed cells with 1 to 5 mmol/L SAM concentration (98% and 85%, respectively) and this effect was time-dependent. Based on this data, we used 1 mmol/L SAM for subsequent experiments.

SAM induces a synergistic antiviral effect with standard IFN treatment but it is independent of IFN signaling

We further investigated whether the effect of SAM on HCV expression is similar to IFN-standard treatment (PEG-IFN + RBV). We incubated Huh7-replicon cells either with 1 mmol/L SAM, PEG-IFN + RBV or combined treatment, and then cells were harvested at three different time points. Real-time RT-PCR assay showed that viral RNA was downregulated in a time

dependent fashion reaching the highest inhibition value at 72 h post-SAM treatment in HCV replicon cells (60% of inhibition at 48 h and 75% of inhibition at 72 h, ^a $P < 0.01$) (Figure 2A). As we expected PEG-IFN + RBV treatment declined HCV-RNA levels at 24, 48 and 72 h upon treatment (around 80%). Interestingly, combined treatment of SAM + PEG-IFN + RBV showed a maximal inhibition of HCV-RNA at the three times of exposition (around 92% of inhibition at 24, 48 and 72 h, ^a $P < 0.01$) (Figure 2A). Taken together, these results demonstrated that SAM possesses a synergistic antiviral effect in this replicon cell culture system.

Next, we evaluated the effect of these treatments on HCV protein expression. Cell lysates were prepared and subjected to western blot analysis to detect NS5A protein. Results from three immunoblots were

analyzed by densitometry and graphics showed similar results (Figure 2B). Basically, we found that NS5A protein levels were also decreased in a time depended manner. We also observed a synergistic effect of SAM + PEG-IFN + RBV treatment to decrease viral proteins at 24, 48 and 72 h (40%, 60% and 55% respectively) ($^dP < 0.01$).

To investigate if this synergic antiviral effect is mediated by a higher stimulation of IFN signaling by SAM, we examined the STAT1 and PKR protein levels by western blot analysis. We found that 1 mmol/L SAM-treatment does not induce STAT1 and PKR protein expression (Figure 2C, lanes 2, 6 and 10) in Huh7-replicon cells at 24, 48 and 72 h. Contrary to this, it seems that SAM decreases STAT1 protein expression at the three times of exposure (Figure 2C, lanes 2, 6 and 10). On the other hand, immunoblot analysis of protein extracts from IFN + RBV or SAM-combined treated cells clearly showed an increase in PKR and STAT1 proteins (Figure 2C lanes 3, 4, 7, 8, 11 and 12). Together these data suggest that interferon signaling seems not stimulated by SAM exposition and therefore, inhibition of viral replication and protein synthesis by SAM may be independent of IFN-induced status.

SAM exposition does not modify viral RNA stability, but it needs cellular translation machinery in order to decrease HCV expression

The aforementioned results suggest the possibility that SAM-treatment may either diminish the transcription rate of viral RNA or decrease viral RNA or protein stability in addition of the negative effect on HCV-RNA levels observed before (Figure 2). To investigate this point we next explored whether SAM modifies HCV-RNA levels upon actinomycin D (Act D) and cycloheximide (CHX) treatment.

Huh7 HCV-replicon cells were treated either with Act D (to block cellular mRNA transcription by RNA polymerase II) or CHX (to block the cellular translation rate of proteins) and 2 h later 1 mmol/L SAM was added. Cells were incubated for sixteen hours and then harvested in order to perform HCV-RNA quantification. We found that in Act D-treated cells, the viral RNA level was stabilized and accumulated (it was not degraded), which explains that after 16 h of treatment there was an increase in the basal levels of HCV-RNA (steady-state levels). When cells were treated simultaneously with SAM and Act D, SAM treatment was not able to decrease increased basal levels of viral RNA stabilized by Act D. On the basis of this finding, we can conclude at this point that the reduction on viral RNA mediated by SAM is an independent mechanism that does not involve alterations in its half-life and reduction of HCV-RNA stability (Figure 3A).

Moreover, in the cells treated with CHX for the indicated times, we observed that HCV-RNA levels were not affected by blocking translational machinery

but upon SAM addition, a slight increase of viral RNA was observed (Figure 3A). Interestingly, when we analyzed the NS5A protein levels upon CHX alone or combined with SAM treatment, we observed that protein levels were slightly decreased at 16, 24 and 36 h upon CHX stimulation compared with control cells (non-treated cells) (Figure 3B). However, when cells were exposed to CHX + SAM, the NS5A protein levels were greater than those observed in cells incubated only with SAM at the same times (Figure 3B). We found that CHX partially reversed the negative effect of SAM on HCV expression (Figure 3B; lane 4, 8 and 12). After a cell is infected by virus, numerous cellular mechanisms are triggered to prevent the virus from completing its viral cycle. Many of these mechanisms depend on the activation and increased synthesis of proteins with specific antiviral activity (as PKR, Mx1, OAS), so when cellular translation is inhibited, the synthesis of these proteins also decreases, then the levels of viral RNA could increase during this period due to the absence of inhibiting replication proteins. Together, these results suggest that cellular translation machinery and protein stability could play a role in the modulation of HCV protein synthesis by SAM in cultured cells.

Effect of SAM exposition on oxidative stress markers

Cells are protected against oxidative damage by natural antioxidant molecules, notably glutathione, and several antioxidant enzymes. It is reported that one of the biological activities of SAM is as antioxidative agent^[23]. However, there is not enough data on whether its antioxidant properties could be implicated in the downregulation of HCV expression. We studied the modulation of oxidative stress markers in the negative regulation of HCV-RNA induced by SAM. First, we were interested in determining whether SAM could influence cellular enzymatic antioxidant systems expression, such as SOD 1 and 2 enzymes, CAT, and TRX1 in order to decrease viral expression. To evaluate this effect, mRNAs levels of these enzymes were examined by quantitative real-time RT-PCR in cells expressing HCV-proteins exposed to 1 mmol/L SAM for 0 to 72 h, and then compared to non-treated cells. We observed that transcriptional expression of SOD-1, SOD-2 and TRX1 was upregulated (0.5-fold, 2.5-fold and 2 fold, respectively compared to control) at different times of exposition (24-72 h) in the same cell system (Figure 4A).

SAM is the main precursor of glutathione synthesis. Next, we measured the total glutathione levels in HCV-replicon cells treated with 1 mmol/L SAM from 0 to 24 h by using Ellman's recycling method. As we expected, we found that total glutathione levels were increased in treated cells upon 6 h post-treatment (about 80%-60%) compared to control cells without SAM, and then levels began to decrease (Figure 4B).

Finally, we measured if these antioxidant mecha-

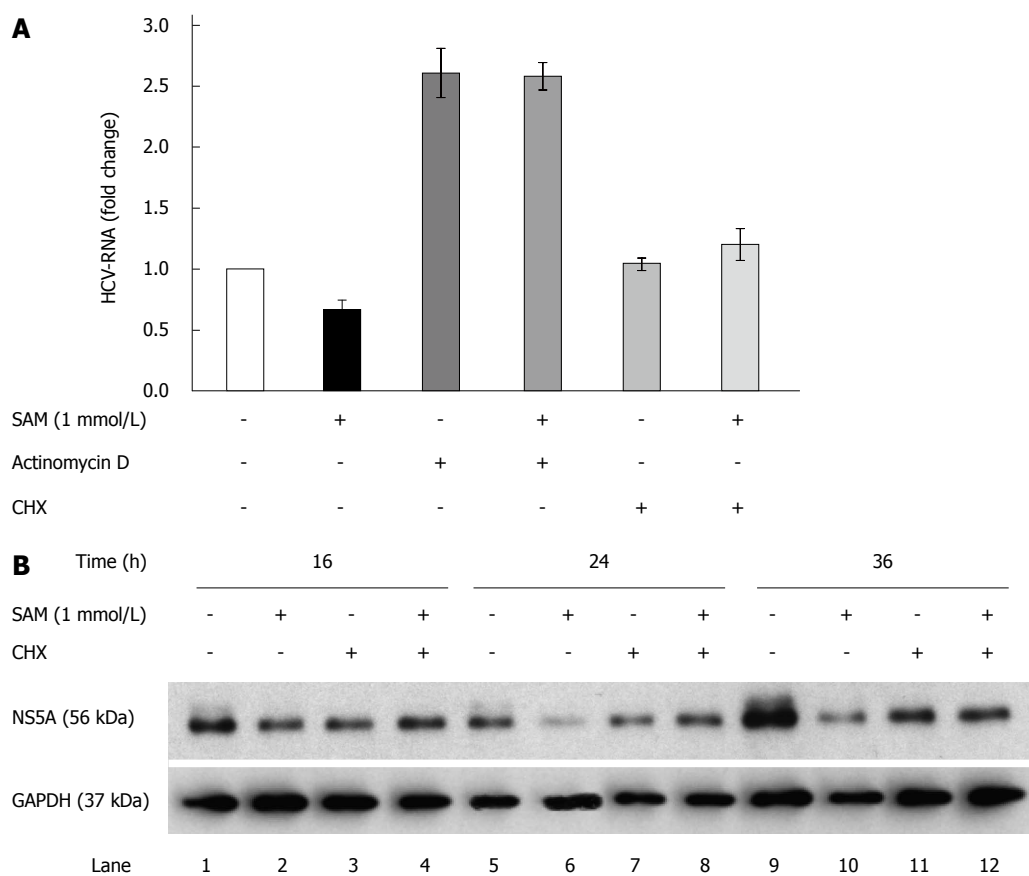


Figure 3 Role of actinomycin D and cycloheximide on the antiviral effect of S-adenosyl-L-methionine. A: SAM decreases replicon RNA levels without affecting its stability. Huh7 HCV replicon cells (1.5×10^5 cells) were incubated with SAM 1 mmol/L, some cells were treated with actinomycin D (4 μ g/mL) or CHX (50 μ g/mL) for 2 h, then SAM was added. Cells were harvested 16 h later and HCV-RNA levels were quantified by real-time RT-PCR and normalized with GAPDH using $\Delta\Delta$ Ct method. Mean results from three experiments are shown; B: CHX partially reverts the downregulated NS5A protein expression induced by SAM. Huh7 HCV replicon cells (1.5×10^5 cells) were incubated with SAM 1 mmol/L, some cells were treated CHX (50 μ g/mL) after 4 h of SAM treatment. Cells were harvested at different time points (0-36 h), then total protein was extracted and western blot for NS5A and GAPDH was performed. CHX: Cycloheximide; SAM: S-adenosyl-L-methionine; HCV: Hepatitis C virus.

nisms up-regulated by SAM are enough to counteract the oxidative stress induced in the cells by HCV proteins and then decrease viral expression. Huh7 replicon-cells were incubated with 1 mmol/L SAM at different time points (0 to 48 h) and then ROS levels were assessed by DCFH-DA assay. A potent antioxidant, pyrrolidine dithiocarbamate (PDTC), was used as a control to decrease ROS^[24] and H₂O₂ was used as a positive damage control. Interestingly, we observed that there was no significant variation in ROS levels in both cell types after SAM exposition at all times evaluated, in contrast to the observed with PDTC exposition where an average of 40% reduction was observed (0.5-24 h) (Figure 4C). Our results suggest that the antiviral activity of SAM could be mediated at least partially by the induction of enzymatic antioxidant systems and total GSH turnover, triggering unknown signaling pathways but with minimal effect on ROS levels.

Turnover of MAT1A/MAT2A is induced by SAM treatment in Huh7 replicon-cells

In normal liver MAT1A is responsible for SAM bio-

synthesis, but upon several liver insults it is replaced by MAT2A in liver regeneration and HCC. Huh7 cells are derived from a hepatocarcinoma cell line, then in our experimental setting it was expected that MAT1A levels were downexpressed and MAT2A were major expressed. To further address the mechanisms involved in the SAM-induced downregulation of HCV expression, we evaluated the role of MAT1A and MAT2A enzymes in our cell system expressing HCV proteins. HCV replicon-cells were treated with SAM as mentioned before and then qPCR and western blot assays were performed to evaluate enzyme expression. From our results we observed two complementary phenomena (Figure 5). First, a transcriptional downregulation was observed in both enzymes at 24 h because cells were plenty of SAM, thus they do not need to synthesize it more; but later on we observed a major upregulation of MAT1A-mRNA at 48 h upon treatment (2.8 fold-times), while MAT2A-mRNA synthesis was less induced (1.4 fold) (Figure 5A). This turnover from MAT1A /MAT2A at 48 h upon SAM exposition is better represented in Figure 5B.

Second, there was a translational upregulation of

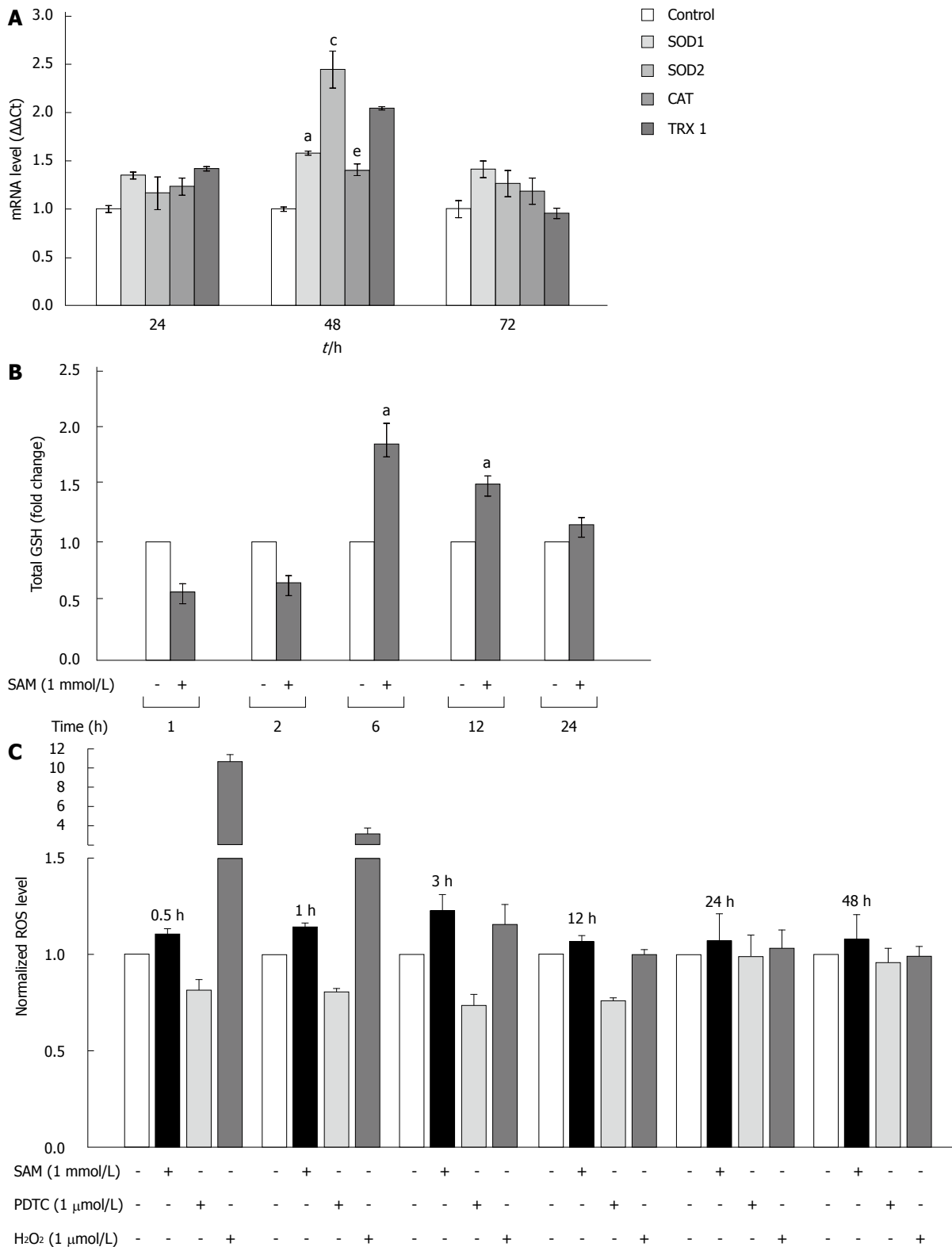


Figure 4 Effect of S-adenosyl-L-methionine exposition on oxidative stress markers. A: Effect of SAM on antioxidant enzyme expression. Huh7 HCV replicon cells (2×10^5 cells) were incubated with 1 mmol/L SAM and then cells were harvested at 24, 48 and 72 h. SOD1, SOD2, catalase and thioredoxin 1 mRNA levels were quantified by real-time RT-PCR and normalized with GAPDH and RPS18 using the delta delta Ct method. Mean results from three independent experiments are shown. ^a $P \leq 0.05$ comparison of SOD1 expression in cells treated with SAM vs untreated cells; ^b $P \leq 0.05$, SOD2 expression of SAM treated cells vs untreated; and ^c $P \leq 0.05$, TRX1 expression of SAM treated cells vs untreated; B: Total glutathione amount in HCV-replicon cells treated with SAM. Huh7 HCV-replicon cells (2×10^5 cells) were treated with 1 mmol/L SAM. Total glutathione level was evaluated at different times by Ellman's recycling method (0-24 h). Mean results from three independent experiments are shown; C: Effect of SAM in ROS levels. HCV replicon-containing cells (2×10^4 cells) were incubated with 1 mmol/L SAM at different time points (0.5, 1, 3, 12, 24 and 48 h). ROS levels were assessed by DCFH-DA assay. Fluorescence was measured at 503 nm and 530 nm, excitation and emission wavelengths, respectively. Hydrogen peroxide (1 μ mol/L H₂O₂) was used as a positive damage control and pyrrolidine dithiocarbamate (5 μ mol/L PDTC) as antioxidant control. SAM: S-adenosyl-L-methionine; HCV: Hepatitis C virus; SOD: Superoxide dismutase; ROS: Reactive oxidative species; PDTC: Pyrrolidine dithiocarbamate.

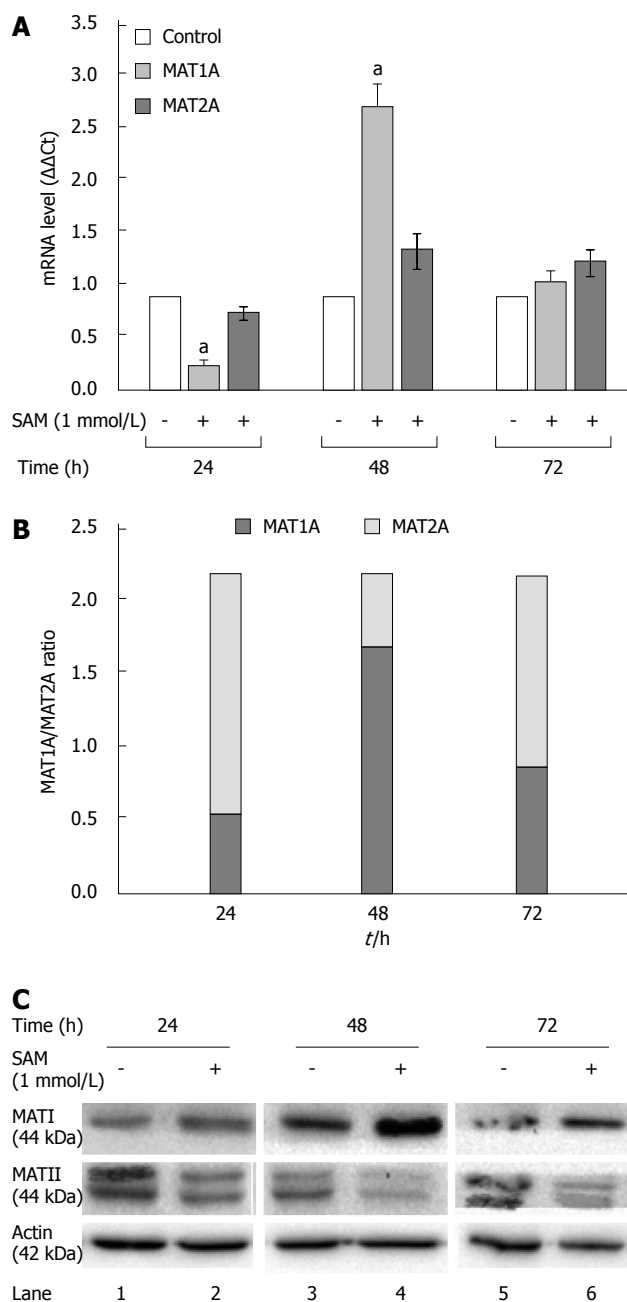


Figure 5 Effect of S-adenosyl-L-methionine on methionine adenosyltransferase 1/2 turnover. **A:** Assessment of MAT-mRNA: Huh7 HCV replicon cells (2×10^5 cells) were incubated with 1 mmol/L SAM and then cells were harvested at 24, 48 and 72 h and MAT1 and MAT2 mRNA levels were quantified by real-time RT-PCR and normalized with GAPDH and RPS18 using delta delta Ct method. Mean results from three independent experiments are shown. $^aP \leq 0.05$ comparison of SAM vs untreated cells; **B:** MAT1A/MAT2A Turnover: Measured MAT1A/MAT2A expression represented as a total; **C:** MAT1 and MAT2 protein expression: Huh7 HCV replicon cells (2×10^5 cells) were incubated with SAM 1 mmol/L. Cells were harvested at 24, 48 and 72 h and total proteins were obtained. Cell lysates were prepared, and equal amounts of protein extracts (40 μ g) were subjected to immunoblot analysis to detect MAT1, MAT2 and actin levels. SAM: S-adenosyl-L-methionine; HCV: Hepatitis C virus; MAT: Methionine adenosyltransferase.

MAT1A protein since 24 to 72 h upon SAM exposition. Contrary to these, MAT2A protein levels were decreased upon SAM exposition since 24 to 72 h compared with untreated cells (Figure 5C).

DISCUSSION

Recently it has been reported that SAM treatment improves early virological response in CHC patients with previous nonresponse treated with PEG-IFN + RBV^[25]. In this study we demonstrated that SAM alone has the capacity to downregulate transcriptional and translational HCV expression in a hepatoma cell line expressing HCV viral proteins. In addition, there are *in vivo* and *in vitro* experiments reporting that SAM could enhance the antiviral effect of IFN, based on that SAM might be related to an early viral response. Our findings reinforce partially the data presented by Feld *et al.*^[26] showing that SAM possesses antiviral action against HCV. In Feld's report the effect *in vitro* of the addition of SAM on viral clearance was not apparent until 72 h after interferon treatment, while in our experiments we found a clear decline of HCV expression since 24 h upon SAM alone or combined (SAM + IFN + RBV) exposition (Figures 1B and 2) and that could be due to the higher dose used in our experiments (1 mmol/L SAM). Contrary to results found by Feld *et al.*^[26], our results reveal that SAM possesses an early synergic antiviral effect when it is added to IFN + RBV treatment in HCV replicon cell culture system.

On the other hand, in our study the addition of SAM to PEG-IFN α and RBV does not improve the expression of ISGs genes. We observed that PKR and STAT1 protein levels were stimulated in the presence of IFN α + RBV but not upon SAM exposition, suggesting that HCV downregulation mediated by SAM is independent of IFN α stimulated pathways. These findings could have clinical application in terms of understanding the pathophysiology of the disease.

SAM is a pleiotropic molecule involved in multiple cellular reactions, participating directly in the following three types of reactions: transmethylation, trans-sulfuration and aminopropylation^[27]. SAM is also involved in many other biochemical reactions in the human body, serving as a key metabolite that regulates hepatocyte growth, death and differentiation^[28,29]. Based on this, we explored if alteration of RNA stability or basal levels are triggered by SAM or its metabolites. Based on our finding, we can suggest that the decrease in viral RNA mediated by SAM is due to an independent mechanism that does not involve alterations in its half-life and reduction of HCV-RNA stability (Figure 3A). However, SAM needs a cellular protein translation process to subsequently decrease the expression of viral proteins (Figure 3B).

It has been well established that SAM is the main methyl donor in methyltransferase reactions and that SAM supplementation restores hepatic glutathione deposits, which is a well-known anti-oxidant important for prevention of liver injury. To investigate the possible mechanism(s) responsible for the SAM anti-HCV observed effect we examined antioxidant enzyme RNA levels and GSH/GSSG ratio. Previously we have

reported that SOD-1, CAT and GPX enzymes expression were already induced in Huh7 replicon cells while it was almost undetectable in parental cells^[30]. In this study we found that SAM up-regulated SOD1, SOD2, and thioredoxin-1 expression; induced total glutathione levels, and decreased HCV-RNA levels (Figure 4A and B) but without modification of ROS levels in Huh7 HCV replicon cells. These findings are difficult to explain because we expected ROS levels could decrease upon antioxidants enzyme and GSH/GSSG were upregulated. There are some explanations for the divergent results. One explanation could be that the site of major expression of these enzymes and turnover ratio is the cytoplasm; and as we previously reported, ROS induced by HCV proteins are mainly produced in the mitochondria^[30]. Another plausible explanation is that antioxidant enzymes are triggering different signaling pathways not affecting ROS amount but decreasing viral proteins. It is possible that additional immediate-early genes are also directly affected by SOD1, SOD2 or TRX and then also affects downstream events. Furthermore, oxidants/antioxidants may have different effects on other steps of the HCV life cycle. The possibility of considering SAM as a modulator of oxidative stress levels is not new. Recent evidence from Brown *et al.*^[23] have demonstrated that an overdose of acetaminophen induces oxidative stress in the whole liver and mitochondrial subcellular fractions which in turn increases cellular levels of ROS and reactive nitrogen species and induces GSH depletion as a critical early events in acetaminophen hepatotoxicity. In cells treated with SAM, they found that acetaminophen hepatotoxicity was blocked by SAM treatment^[23]. These data suggest that the antioxidant status was compromised with several important components of the antioxidant defense mechanism being significantly decreased or increased in HCV-infected cells.

These findings provide new clues to further explain the mechanism(s) that indirectly suppressed HCV replication; however, the mechanism(s) still are poorly defined and additional approaches should be designed.

The molecular mechanism of HCV pathogenesis remains unclear but it is well known that both viral and host factors contribute to disease outcome. MAT is an essential cellular enzyme that catalyzes the formation of SAM. In mammals, two genes (*MAT1A* and *MAT2A*) encode homologous MAT catalytic subunits which are differentially expressed among different tissue^[16]. *MAT1A* encodes for $\alpha 1$ that forms dimer (MATIII) and tetramer (MATI) that are predominantly present in liver parenchymal cells; while *MAT2A* encoding the $\alpha 2$ catalytic subunit of the MATII isoenzyme that is expressed in all other tissues. In patients with chronic liver disease there is a decrease of SAM biosynthesis exacerbating liver injury, and then exogenous supplementation might represent a useful therapy^[17,31,32]. However, the effectiveness of SAM treatment in chronic liver disease has not been adequately studied.

Results found in this study suggest that a possible

mechanism by which SAM decreases HCV expression could involve an additive effect between modulation of antioxidant enzyme systems, glutathione restore levels (GSH/GSSG) and switching MAT2A/MAT1A turnover in treated cells, because all these molecules participate in cell surviving pathways. In addition, Feld *et al.*^[26] found that STAT1, a transcription factor responsible for antiviral/IFN signaling activation, was methylated in cells treated with SAM, so, in that way, SAM could be available to translocate to the nucleus and do its function. Together these results provide new clues to explain the mechanism(s) involved in the antiviral effect of SAM against HCV. In addition, a fine balance of the MAT1A/MAT2A ratio induced by SAM should play a role in HCV replication and in the modulation of antioxidant enzymes. Therefore, if SAM can modify oxidative stress systems, glutathione restoring and MAT1A/2A cell signaling pathways, these activities can provide hepatocytes with the capacity to counteract the damage induced by HCV infection.

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COMMENTS

Background

S-adenosyl-L-methionine (SAM) is a pleiotropic molecule that is involved in multiple cellular reactions, participating directly in the following three types of reactions: transmethylation, trans-sulfuration and aminopropylation. SAM is also involved in many other biochemical reactions in the human body, serving as a key metabolite that regulates hepatocyte growth, death and differentiation.

Research frontiers

It has been reported that SAM treatment improves early virological response in chronic hepatitis C subjects with previous nonresponse treated with PEG-IFN + RBV, while *in vitro* decreases the levels of hepatitis C virus (HCV) RNA, however the implicated mechanisms are unknown.

Innovations and breakthroughs

Results from this study suggest that SAM is able to diminish HCV expression at least in part through modulation of antioxidant enzymes, biosynthesis of glutathione and switching methionine adenosyltransferase 1A (MAT1A)/MAT2A turnover in HCV expressing cells.

Applications

These findings suggest the possibility that SAM modulation could help to counteract the HCV-induced damage.

Terminology

HCV infection is a major global health issue, with an estimated 3% of the world's populations being chronically infected. HCV is a noncytotoxic hepatotropic member of the *Flaviviridae* that causes acute and chronic hepatitis, and hepatocellular carcinoma. It has been shown that HCV modifies antioxidant defense mechanisms yielding a cellular oxidative imbalance and further cell death.

Peer-review

The authors investigated the antiviral effect of SAM with a HCV subgenomic replicon cell culture system. It's a well written paper with clear hypothesis, the

data is solid and well presented.

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